From Coiled-coil Chemistry to Muscle Contraction (Commemoration Issue Dedicated to Professor Tatsuo Ooi, On the Occasion of His Retirment)

AUTHOR(S):

Ueno, Hitoshi

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Review

From Coiled-coil Chemistry to Muscle Contraction

Hitoshi UENO*

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Proteases are widely used in protein chemistry. Since proteolytic digestion of proteins can be treated by the Michaelis-Menten equation, the cleavage rate constant is found to be interpreted as reflecting local structural changes. The cleavage rates as well as sites are determined on SDS-containing gel electrophoresis. The quantitative enzyme-probe method and its application are reviewed.

KEY WORDS: Limited proteolysis/ Enzyme-probe method/ Myosin/ Subfragment-2/ Helix-coil transition/

INTRODUCTION

It is a particular pleasure to share an opportunity to contribute to this special issue. Since I was a graduate student in Prof. Ooi's laboratory, I have been interested in physico-chemistry of coiled-coil molecules. Tropomyosin is a typical coiled-coil and was a subject for my Ph. D Thesis. Tropomyosin is rather simple in structure, and extensive protein chemistry involved to elucidate the structure and function relationship of this protein. A coiled-coil molecule may be considered as stable and rigid in general. However, many proteins contain rigid and loose parts[1] within themselves and so does a coiled-coil protein. The loose parts often play important roles in physiological function, which is a major reason, if not all, why proteases such as trypsin[2,3] and carboxypeptidase[4] were used to study troponin-binding and end-to-end polymerizing functions of tropomyosin, respectively.

Although *qualitative*, a use of proteases to investigate the loose parts within a protein was a well-known method[5]. It is fair to say, especially in this particular occasion, that during my graduate course I wished to establish a *quantitative* method to detect local motile regions in a macromolecule by the use of protease(s), and this idea kept in back of my head for several years. The *quantitative* enzyme-probe method blossomed later while I enjoyed very much my research carrier with Prof. Harrington at the Johns Hopkins University, Baltimore, Maryland, where I also learned a coiled-coil segment of myosin could create motility and excitement. Although my major interest has been an elucidation of the origin of contracitle mechanism, I would rather emphasize the enzyme-probe method to pay my special regards to both Prof. Ooi and Prof. Harrington.

* 上野 均: Ube Laboratories, Ube Industries, Ltd., Ube, Yamaguchi 755

ENZYME-PROBE METHOD

In this presentation proteolytic enzymes are used to probe the stability of local structural domains in coiled-coil molecules. The enzyme-probe method[6] as described in this article monitors cleavage by following the time-dependence of the decay of a substrate protein as well as the rate of formation and the size of proteolytic fragments on SDS-containing gels. Samples of digestion mixture were removed at various reaction times and proteolysis quenched by addition of appropriate inhibitor. These samples were then denatured in the presence of SDS and run on SDS-containing gels. Absorbance of each peptide band was determined by densitometry of the gels and further reduced to a relative molar ratio using the molecular weight of each polypeptide estimated from its electrophoretic mobility in SDS-containing gels according to Weber and Osborn[7].

Digestion of a protein with proteolytic enzymes can be treated by application of the Michaelis-Menten equation[5].

$$-\frac{d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_m+[S]} \qquad (1)$$

where $[S]$, $[E]$, k_{cat} and K_m represent substrate, enzyme, catalytic rate constant and the Michaelis-Menten constant, respectively. If $K_m \gg [S]$, then eq(1) can be simplified as follows:

$$-\frac{d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_m}. \qquad (2)$$

For this equation, $k_{cat}[E]_0/K_m$ represents the apparent rate constant k_{app} which is obtained from a plot of $\log[S]$ against time. Under the experimental conditions we employed, K_m was more than 10 times larger than $[S]$. In other words, log(absorbance) *versus* time plots for the substrates were linear under the present conditions. The pseudo-first-order rate constants of cleavage thus obtained are normalized for the intrinsic hydrolysis rate of the enzymes using low moleculular weight model esters. This normalization will minimize the effect of various experimental conditions such as temperature, ionic strength, solutes and others on the intrinsic enzyme activity.

From eq(2), the apparent rate constant k_{app} is proportional to enzyme concentration $[E]_0$ but not to substrate concentration $[S]$. Figure 1 shows chymotryptic digestion of rabbit skeletal muscle myosin rod to examine the effects of substrate and enzyme concentrations on the cleavage. Densitometry recordings of the gels reveal that the digestion patterns were closely similar despite the several fold difference in substrate concentration (Fig. 1(a)) and that when an invariant concentration of myosin rod was digested at differing concentration of chymotrypsin, the digestion patterns were again closely similar at comparable stages of cleavage (Fig. 1(b)). Digestion rate constants derived from plots of undigested rod *versus* digestion time showed a gingle exponential decay and were independent of the substrate concentration but proportional to enzyme concentration (Fig. 1(c)).

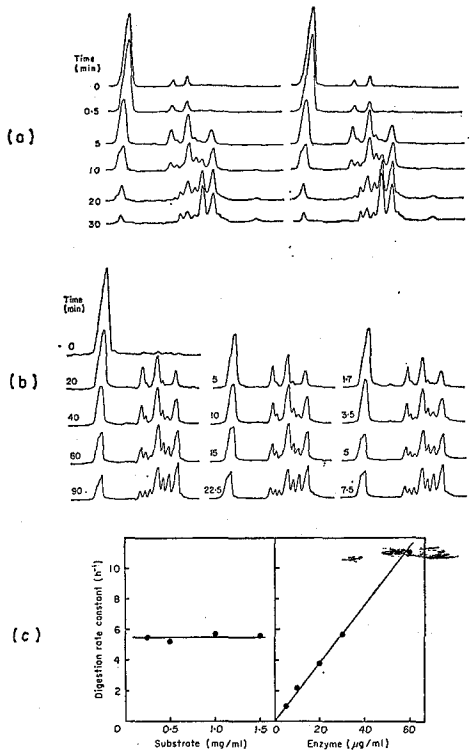


Fig. 1. Chymotryptic digestion of rod subfragment of rabbit skeletal muscle myosin[6]. (a) Myosin rod (0.25 and 1.5 mg/ml for left and right, respectively) was digested with chymotrypsin (30 μg/ml) at 20°C and pH 7.1 (μ=0.5). Densitometric tracings of SDS-containing gels of rod digest at each digestion time are shown. (b) Time-dependence of densitometric recordings of the gells on SDS-containing gels. Digestion times are given. Myosin rod (1.0 mg/ml) was digested at chymotrypsin concentratin of 5, 20 and 60 μm/ml from left to right, respectively. (c) Enzyme-substrate relationship in the enzyme-probe method. Substrate (myosin rod) and enzyme (chymotrypsin) ratios were varied to compare digestion rates. (Left) Substrate concentration was varied with a fixed enzyme concentration as in (a). (Right) Chymotrypsin concentration was varied with a fixed concentration of enzyme as in (b).

MODEL SUBSTRATES

To see validity of the enzyme-probe method, two model polypeptides, ribonuclease and coiled-coil tropomyosin fragment, were compared in the kinetics of tryptic cleavage in their folded and unfolded states (Fig. 2). It is well-known that native ribonuclease is a compact globular protein highly resistant to proteolysis under physiological conditions whereas reduced alkylated ribonuclease is an unfolded structure with the physicochemical properties of a random coil[8]. Figure 2(a) shows the normalized tryptic digestion rate constant (in h^{-1}/units) for ribonuclease is virtually zero over the temperature range 10 to 40°C, while that for alkylated ribonuclease is about 100 h^{-1}/units. Figure 2(b) shows the normalized rate constant of tryptic cleavage of a tropomyosin fragment in an ionic strength-dependent two-state equilibrium between a two-chain, α-helical structure and a random coil. The rate

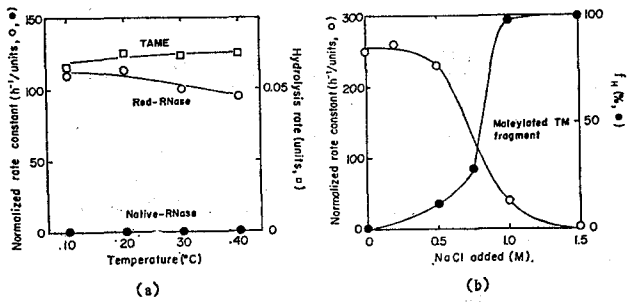


Fig. 2. Tryptic digestion of model polypeptides[6]. (a) Temperarure-dependence of tryptic cleavage rate constants of TAME (p-toluenc toluenesulfonyl-L-arginine methyl ester hydrochloride), native and reduced alkylated ribonucleases. (b) Salt-induced conformational transition in maleylated tropomyosin fragment. Tryptic cleavage rate constants (open curcles) and fraction of α-helical constant (filled circles) *versus* NaCl concentration are shown.

constant for the folded state was about 250 h^{-1}/units and that for the unfolded state virtually zero.

The two examples in Figure 2 indicate that the enzyme digests the unfolded form at a markedly higher rate than it digests the folded form more than three orders higher in magnitude. The proteolytic enzymes seem to probe the loose state or region(s) of a substrate protein.

ENZYME-PROBE OF MYOSIN ROD

In our earlier work we[9] used chymotrypsin to probe local conformational changes within the α-helical subfragment-2(S-2) region of the myosin cross-bridge when glycerinated muscle fibers were activated to contract. This rather qualitative chymotrpsin-probe study was no doubt motivated by the earlier theoretical considerations of Prof. Harrington,[10,11] and encouraged us to extend our work towards our following quantitative enzyme-probe studies[9,12-16]. There was considerable evidence that the myosin rod is flexble at or near the protease-susceptible region studied by the use of various methods. A great advantage of the enzyme-probe is to pinpoint the local unfoled region. For example, proteolytic fragments observed after digestion on the gels will provide us with the precise cleavage site(s) if the location of the fragments are properly assigned within the original undigested polypeptide.

The temperature-depepdence of proteolytic cleavage of the myosin rod was investigated over the temperature range 5 to 40°C. It is worth noting that three different enzymes (chymotrypsin, trypin and papain) were used to probe the rod to confirm that the observed conformational state and/or change as revealed by this probe method is virtually independent of the enzyme species used.

When the proteolytic digests of the rod at varoius temperatures were analysed on SDS-containing gels, it was apparent the cleavage fragmentation patterns are different at various temperatures. At lower temperatures (<25°C), a small number of discrete proteolytic fragments were observed in the digestion pattern of each enzyme, whereas at higher temperature (>30°C) a rather broad distribution of many weak

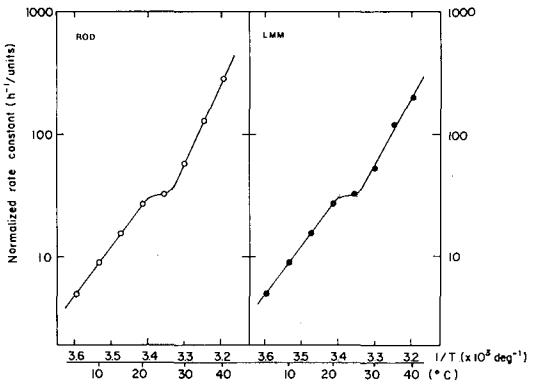
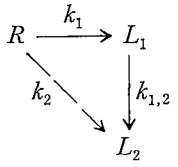


Fig. 3. Arrhenius plots of papin cleavage of myosin rod and formation of LMM subfragmens. All rate constants are normalized as described previously[6].

bands observed.

The normalized cleavage rate constant, k, *versus* temperarure profiles obtained for the three enzymes were roughly similar: a small but distinct shoulder was observed between 20 and 30°C, followed by a sharply increasing rate constant as the temperature was elevated above 30°C. This distict presence of two stages below and above 25°C is more clearly observed in Arrhenius plots (log k *versus* $1/T$) of the digestion rate (Fig. 3).

Since the temperature-dependent structural changes detected by enzyme-probes appeared to be localized in the hinge domain of the myosin rod, this temperature-dependent cleavage was also investigated by following the kinetics of formation of discrete proteolytic fragment(s), LMM(light meromyosin). In order to obtain the time-course of appearance of proteolytic fragment(s), the following scheme of fragmentation was assumed:



where R, L_1, and L_2 represent undigested rod, high molecular weight LMM fragment(s) and low molecular LMM fragment(s), respectively. k_1, k_2 and $k_{1,2}$ are first-order rate constants. More details are described previously[9]. Figure 3 compares Arrhenius plots of the digestion rate of the myosin rod with the rate of formation of the LMM fragments. The two plots show two major stages of digestion with a well-defined transition between the stages at 25 and 30°C. It should be noted that qualitatively similar Arrhenius profiles were seen in three different enzyme (chymotrypsin, trypsin and papain) digestion studies. At temperatures between 5 and 25°C, the apparent heat of activation $\Delta H^{\ddagger}$=18–23 kcal/mol, while $\Delta H^{\ddagger}$=30 kcal/mol between 30 and 40°C. This similar temperature-dependent profile for all three enzymes used in this study suggests that they are probing localized melting of the

α-helical, coiled-coil structure of myosin rod. Although it seemed clear from the digestion profiles over the wide temperature range that the proteolytic cleavage sites are localized within a narrow region at lower temperature (<25°C) and spanning a much broader region at higher temperature (>25°C), it was rater difficult to pinpoint the exact cleavage sites, particularly at higher temperature. We, therefore, decided to use well-defined S-2 subfragments of myosin in our following study in order to establish the location of the melting sites and the thermal stability of the local regions.

MYOSIN S-2

Temperature-dependent structural changes in the S-2 region were investigated using isolated S-2 subfragments, Three well-defined S-2 subfragments were prepared as shown in Figure 4: two long S-2 (53,000 Mr and 61,000 Mr) and a short S-2 (34,000 Mr) fragment. The two long S-2 fragments contain the hinge domain and allow a determination of the cleavage rate(s) within this region. A comparison of cleavage rates within this hinge region of these two long S-2 fragments also provides information on the "end-effect" on the structural integrity of the hinge domain.

The time-course of the fragmentation of the two long S-2 species were followed using three different enzymes as before to probe the strutural changes specifically within the hinge domain. As described previously in details,[12] proteolytic digests of the S-2 were quenched at various reaction times and the rate of formation (or decay) of the proteolytic fragments monitored on SDS-containing gels followed by simulating the digestion kinetics using a computer.

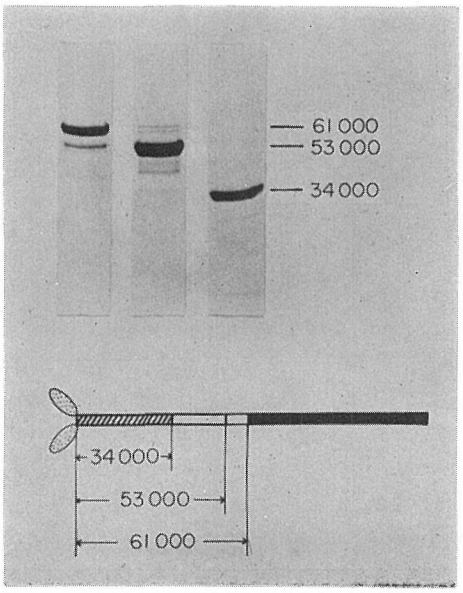


Fig. 4. Subfragment-2 prepared from rabbit skeletal muscle myosin used in our study[12]. SDS-containing electrophoresis of three different S–2 subfragments are shown. Schematic representation of the location of the S–2s in the myosin molecule is also shown (bottom).

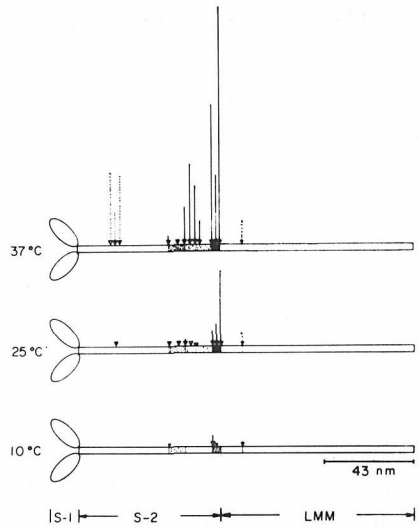


Fig. 5. Schematic representation of proteolytic cleavage in the S–2 region of a myosin molecule[12]. Normalized cleavage rate constants at various regions are presented relative to digestion rate constants for the S-2 at 40°C. Lengths of arrows are proportional to relative rates of cleavage. Digestion conditions: μ=0.15, pH 7.3. Chymotrypsin, trypsin and papain were used for the digestion.

The results are schematically illustrated in Figure 5. Although the absolute cleavage rate constants are different, even after they are normalized, among the three proteolytic enzymes, the cleavage profiles at each cleavage site are independent of the enzyme species employed. This supports that the cleavage profiles are truly reflecting local melting of the α-helical sturcture. The two long S-2 fragments exhibited very similar cleavage profiles at each susceptible site, which suggests that the "end-effect" is small if any.

HELIX-COIL TRANSITION

The present results are consistent with the general conception that α-helical,

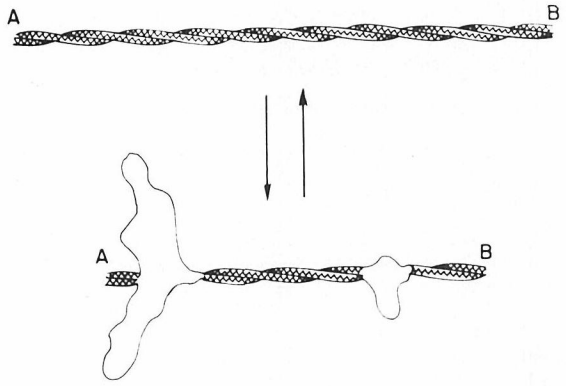


Fig. 6. Schematic illustration of the two-state equlibrium between α-helical (top) and *locally* melting (bottom) states. It is assumed a coiled-coil molecule contains quasi-independent co-operative melting blocks.

coiled-coil molecules are made up of quasi-independent co-operative blocks,[17,18] which is schematically shown in Figure 6. The idea is that a coiled-coil molecule contains several blocks with different thermal stability. Less stable blocks (regions) will melt prior to other stable blocks. Proteases are, therefore, digesting melting blocks under physiological conditions.

The kinetic equation for enzymatic cleavage is considered[16] to be analogous to that describing hydrogen exchange of proteins. Schematically

$$N_A \underset{k_b}{\overset{k_a}{\rightleftharpoons}} U_A \xrightarrow{k_m} P_A \qquad (3)$$

where N_A, U_A, and P_A are concentrations α-helical state, random-coil state, and cleaved product for region A, respectively. Since the relaxation time for reequilibration of the coiled-coil molecule in helix—coil transition is on the order of $10^{-4}\sim10^{-2}$s, k_a and k_b are at the very least $10^5\,h^{-1}$ for a coiled-coil molecule in solution and k_a and $k_b \gg k_m$. Therefore, the cleavage process (k_m) is rate limiting. Then, eq (3) gives the cleavage first-order rate constant αk_m where α is given by $k_a/(k_a+k_b)$. The cleavage rate of any structure is proportional to the fraction of time that the region spends in the open state when the cleavage step (k_m) is rate limiting.

MYOSIN IN MUSCLE

Since the quantitative enzyme-probe method seems to be well-established using several different systems, we are ready to investigate local melting in actively contracting muscle fibers extending earlier preliminary enzyme-probe investigation[9] of

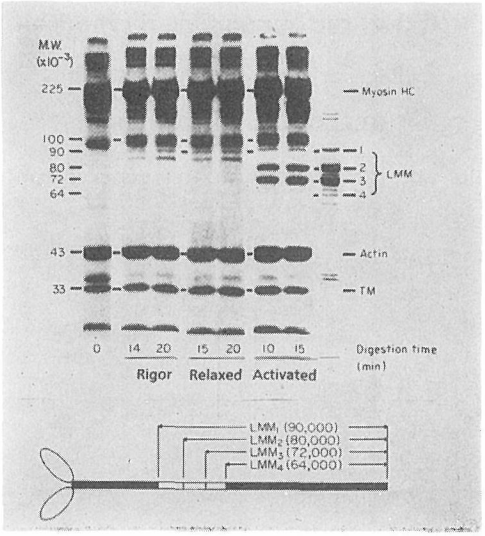


Fig. 7. Electrophoresis of muscle proteins on SDS-containing gels after chymotrytic digestion of glycerinated rabbit psoas muscle fibers[14]. The fibers were preincubated in rigor solution (pH 7.3, μ=0.15) containing creatine phosphokinase and chymotrypsin at 0°C for 3 min. The fibers were then transferred into various digestion solvents. The digestion carried out at 25°C are shown. Location of LMM species is illustrated in the lower scheme.

activated fibers.

Figure 7 shows SDS-containing gel electrophoresis patterns of glycerinated rabbit psoas muscle fibers digested by chymotrypsin. The muscle fibers treated in rigor, relaxing and activating solutions are compared. As was observed,[9] the myosin molecule is highly susceptible to chymotrypsin upon activation and intense LMM bands are observed, whereas no significant proteolysis occurred in non-actiavting (rigor and pseudo-relaxing) solvents. Four major cleavage sites are illustrated in Figure 7(b), and they correspond to 34,000, 44,000, 53,000 and 61,000 Mr S-2 sites. The time-course of chymotrypsin cleavage at 25°C of glycerinated fibers is shown in Figure 8, where the extent of digestion is plotted against digestion time. The cleavage rate for activated fibers in the presence of ATP-regenerating system is

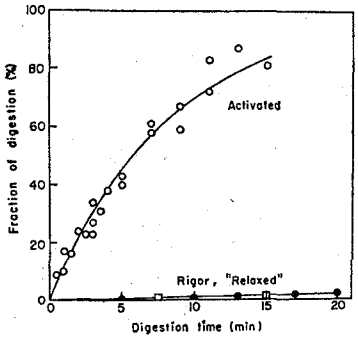


Fig. 8. Time-course of chymotryptic digestion of glycerinated fibers[14] at 25°C and pH 7.3. The relative molar intensity of LMM fragments was used to estimate the extent of digestion and plotted against digestion time. Continuous lines are theoretical curves using rate constants 7.0 h^{-1} (top) and 0.06 h^{-1} (bottom).

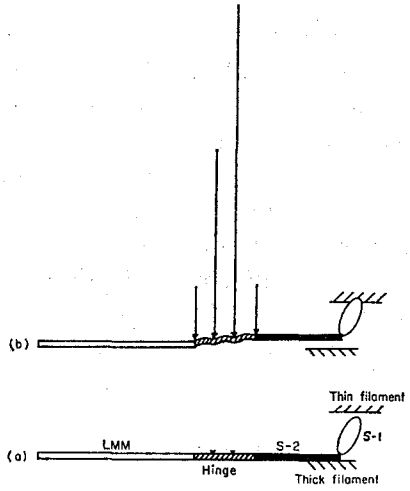


Fig. 9. Schematic representation of chymotryptic cleavage in a myosin molecule within glycerinated rabbit psoas muscle fibers[14]. Normalized cleavage rate constants are compared between (b) activated muscle and (a) relaxed muscle at 37°C. Length of arrows is proportional to the rate constant.

about 100 times (or more) larger than that for non-activating fibers (see Fig. 9).

The temperature-dependence of the normalized rate constants showed that a striking amplification of the local melting within the S-2 hinge domain on activation of muscle fibers occurred in a myosin within glycerinated muscle fibers. The cleavage sites are precisely localized within the hinge domain as is also defined in Figure 5, and the rates 100 times increased upon activation.

Since contractile force depends on MgATP concentration in such a way that force vs −log[MgATP] plot shows a bell-shaped profile with a maximum force at around 0.1 mM-MgATP in the presence of ATP-regenerating system, we compared the chymotryptic cleavage rates at various MgATP concentrations expecting the rates clesely follow the force vs [MgATP] plot. The results showed chymotryptic cleavage rate vs −log[MgATP] plot was superimposed with isometric force vs −log [MgATP] plot. This indicates there appears to be a close linkage between the force generated by actively cycling cross-bridges and the fraction of bridges undergoing melting in the S-2 hinge domain.

MUSCLE CONTRACTION

Earlier proteolytic digestion studies of isolated myosin[20] revealed the existence of two parallel first-order reaction classes with markedly different rates of cleavage. About 10% of the mass of myosin molecule was rapidly released as low molecular weight peptides in the fast reaction. This earlier study[20] is based on the kinetic analysis of enzymatic digestion using pH-stat. Our present studies are more focusing on the S-2 hinge domain which is thermally labile region as is suggested in the earlier work.[20] It seems a great advantage for the present enzymeprobe technique that the enzyme cleavage reaction processes as well as cleavage sites are visualized on the gels.

An α-helical crystalline segment will shorten its end-to-end distance and exert force as it melts to an amorphous random coil state, which is fairly well-established.[21] In fact, the isolated rod is known to shorten while melting whithin the S-2 hinge domain.[22] It is also shown that glycerinated muscle fibers can generate force[23] under rigor solution conditions at high pH where the myosin cross-bridges are known to be released away from the thick filament core and the S-2 hinge domain melted.[24] These results, together with the recent findings that chemical cross-linking of cross-bridges[25] and anti-S-2 antibody binding[25] within muscle fibers can suppress isometric force without abolishing actin-activated MgATPase, suggest that the melting within the S-2 hinge domain can generate force comparable to actively cycling cross-bridges.

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